FREE-ENERGY RELATIONS AND CONTRACTION OF ACTOMYOSIN [1]

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There are two approaches to muscle. One is that of the physiologist, who studies function hoping to understand the nature and reactions of minute structural elements. The other is that of the biochemist, who studies minute structural elements hoping to understand function. The physiologist carefully preserves structure and subtle qualities; the biochemist wilfully destroys them. This destruction may go as far as the dissolution of the system into single molecules. This approach was that of the author's laboratory, which has shown that the contractile matter of muscle is built of two proteins, actin (F. B. Straub 1942, 1943) and myosin.

Destruction need not necessarily go that far. It may be limited to partial dissolution, which leaves the contractile matter and its geometry untouched, or it may simply consist in the disturbance of certain equilibria by thermal or chemical means. In these cases, it is still convenient to call the system "muscle," but it should be clearly understood that by using this word no attempt is made to confuse such a partial system with the whole living and intact machinery.

In themselves, neither myosin nor actin is contractile. If brought together in a suitable ionic milieu they unite to a complex: "actomyosin." According to the concentration and the nature of ions present, the actomyosin may be charged by the ATP and dissociate reversibly into its two components, or else it may be discharged and dehydrated excessively. If this reaction takes place in a heterogeneous suspension, the actomyosin is precipitated. Owing to its violence, this precipitation was termed "superprecipitation" to distinguish it from the weaker dehydration and precipitation induced by salts alone in absence of ATP. If this reaction takes place in an actomyosin gel, it will lead to excessive shrinking, syneresis. If the elongated actomyosin particles are oriented, the shrinking will be anisodiametric and the gel shrinks in the direction of the axis of the particles and expands at right angles to this direction. Actomyosin threads or muscle fibres, under these conditions, may become shorter and wider without changing their volume. If the reaction takes place in the muscle fibre where the elongated actomyosin filaments form a continuous system, the shortening will be able to do work by lifting weights, or develop tension under isometric conditions, and is usually called "contraction."

The study of these phenomena suggested (see Szent-Györgyi, 1947) that the

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contractile matter is built of functional units, "autones," and that contraction is an "all-or-none equilibrium reaction" of these autones, dependent on temperature.[2] Contraction, *i.e.* the dimensional change, in all probability, is secondary to another change in which charges are neutralized. The size of "autones" is independent of the colloidal particle size (1–1, 5×10^6 g) into which myosin breaks up on extraction, and can be expected to be much smaller than this latter. Supposing that the actual shortening is proportional to the number of reacting units, the relative number of charged and discharged units (that is the equilibrium-constant K) was calculated from the macroscopic length of the system.

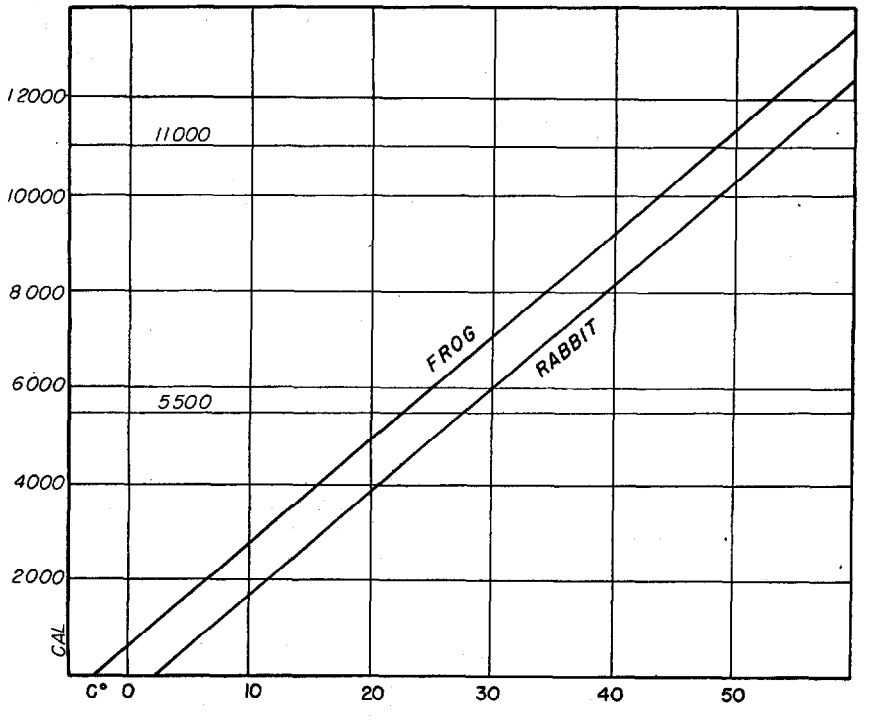

FIGURE 1. The △ F curve of the contraction of extracted frog and rabbit muscle and of frog and rabbit actomyosin threads. Ordinate: △ F in calories. Abscissa: temperature in centigrades (quoted from L. Varga).

From the temperature-dependence of K, the △ F, i.e. the difference of the free-energy content of contracted and relaxed autones, was calculated. The final results of these calculations were summed up by L. Varga (1946) in a curve reproduced in Figure 1. The curve shows that the free energy of the system drops in contraction and that the extent of this drop depends on temperature. In the rabbit it reaches 11,000 calories at 53° C., is 7–8000 calories at 37°, and is 0 at about 0° C.

[2] Buchtal and Knappeis pointed out in 1943 that certain mechanical features of muscular contraction are in accordance with the assumption that the fibre is built of smaller units contracting in an "all-or-none" fashion.

In the frog the same values were reached at 5° C. lower. The curve shows that the relaxed state is the high-energy metastable state, the contracted state the low-energy stable state, contraction being a spontaneous process.

In the first part of this paper material and methods will be discussed. In the second part, the theory will be tested along different lines, and in the third part, the observations will be extended.

Part I: Material and Methods

Muscle is a very heterogeneous tissue. Not only are there different kinds of muscle (smooth, heart and cross-striated muscle), but there are considerable differences between the different muscles of the same sort within the same animal.

There is considerable difference in geometry between the various body muscles. In one muscle the fibres are parallel, while in others they follow a more complicated course, making evaluation of energy relations difficult.

There is considerable difference, also, in the composition of various muscles. The contractile matter, actomyosin, is in its relaxed condition a soft gel which could easily be damaged by mechanical injury were it not protected by connective material, fasciae, collagen fibres and a sarcolem. Muscles lying closer to the surface will need more protection, and in these we will find strongly developed connective material and sarcolem.[3] An almost ideal material for the study of the contractile matter is the musculus psoas of the rabbit. This muscle lies sheltered in the body cavity, protected on one side by the vertebral column, and by the viscera of the belly on the other. Consequently, it contains very little connective tissue, and the sarcolem is poorly developed, which makes the elastic properties of the contractile matter come to the fore. It is built of very long, parallel fibres, stretching from one end of the muscle to the other—fibres which, owing to the poverty of connective material, can easily be separated. It is easy to secure from a medium-sized rabbit very thin fibre bundles 8–10 cm. long which, if necessary, can be decomposed into single fibres of this length. Though occasionally frog sartorius and rabbit m. gracilis were also used, the major part of the experiments reported here were performed on the psoas.

According to the theory outlined, contraction is a spontaneous process going hand-in-hand with a drop of free energy. Thus, contraction should occur spontaneously wherever the ATP-actomyosin system is present in a suitable ionic milieu, and the system should persist in the low-energy stable contracted state. This is actually what happens any time we add ATP to an actomyosin gel or to muscle extracted with water. In the intact resting muscle, however, we find ATP in an active form, linked to actomyosin (see below), but still the system does not contract—contraction being inhibited by some unknown mechanism. If we want the muscle to go over into the contracted state, we have to abolish this inhibition. In the intact muscle this can be achieved by an electric shock or a "wave of excitation." These actions are fleeting and depend on subtle qualities of muscle, on "excitability," which makes them unfit for our present purpose. In order to study equilibria of energy relations, the inhibition had to be removed permanently and uniformly

[3] Ramsey and Street (1940), working with single fibres of the musculus semimembranosus of the frog, found the elastic properties of the contractile matter in resting muscle entirely covered up by the elastic properties of the sarcolem.

throughout the whole mass of the muscle, and the whole contractile matter made to go over into and remain in the contracted state. Poisons like caffeine, quinine, monojodo-acetic acid or chloroform, known to produce contracture, were found unsatisfactory because the tensions developed are very small, showing that only a small fraction of the contractile substance is at any time in the contracted state.

A satisfactory method of abolishing inhibitions is freezing with subsequent thawing, which method also has the advantage that the muscle can be kept in the frozen state, packed in dry ice, for days with undiminished contractility.

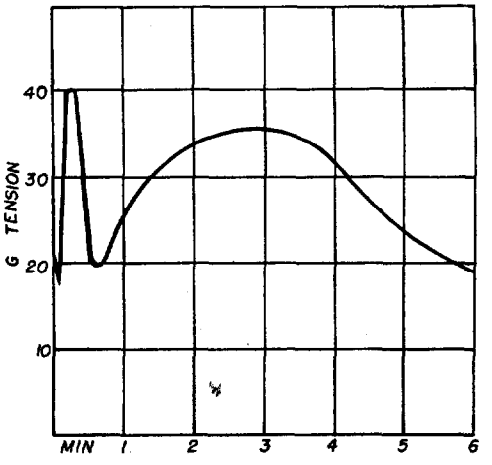

FIGURE 2. Isometric contraction of the frozen sartorius of the frog on thawing. The frozen muscle was immersed into Ringer of 20° C. at 0 min.

The experimental procedure was the following: The rabbit was killed by decapitation, quickly skinned, eviscerated, and the front and the sidewalls of the belly cut off. This exposed the psoas which was liberated from its surroundings. The muscle was decomposed into smaller bundles by punching it through with a small forceps with closed tips and moving the forceps up and down while the index finger of the other hand kept the muscle somewhat lifted. If necessary, ligatures were put on the two ends of the muscle strip. Owing to the poverty of connective tissue, the muscle is rather soft and is easily cut through by ligatures. For this reason, relatively soft and thick threads were applied in dry condition (pearl cotton No. 5).

If frozen strips were desired, fibre bundles of 2–3 mm. diameter were secured, placed on a celluloid ruler (to which the muscle does not stick), stretched to their rest length, the ligature being fixed by artery clamps. Then the strips were covered with freshly powdered dry ice. The strips used were mostly of the thickness of an average frog sartorius, weighing about 40 mg. per cm. Since at higher temperatures the muscle, after freezing, is rapidly damaged, it is important that it should reach temperature equilibrium quickly. So if experiments had to be performed above 30° C., even thinner strips were used weighing 25 mg. per cm.

On thawing, the frozen muscle, if containing the physiological amounts of ATP, contracts rapidly and develops maximal tension.

There are two phenomena which tend to disturb measurements. If the frozen muscle is suddenly placed into Ringer of room temperature, one side may thaw faster than the other and contract suddenly, which causes strong bending which damages the fibres. For this reason, the muscle was allowed to thaw first in 0° Ringer before being transferred into a warmer Ringer of more than 15° C. The first movement of the lever indicates complete thawing which may take place in ten to sixty seconds.

The contraction, elicited by the freezing and subsequent thawing, is developed relatively slowly while the sudden change in temperature may act, in itself, as an impulse and elicit a fast contraction. In this way, in frog muscle, a double peak is obtained, the second of which is mostly lower than the first (Fig. 2). In rabbit psoas this double contraction is less pronounced but still present. Below 10° C. "excitability" is low and the two waves fuse. At higher temperatures they can be made to fuse by making the temperature change less sudden by allowing the muscle to thaw in Ringer of 0° C. before applying the higher temperature Tension given to the muscle also promotes fusion of the two peaks. The same is favored, also, by a thinner diameter. Correct values of maximal work or tension can be obtained only if the two contractions fuse into one.

In order to show whether an observed effect was actually due to an interaction of ATP and actomyosin, the latter had to be prepared free of ATP. The effects observed on addition of ATP could then safely be ascribed to an interaction of the protein and the nucleotide.[4]

Thus muscle fibres had to be prepared, free of ATP, and made permeable to this substance, without destroying the actomyosin structure. In earlier work this was done by extracting the muscle with water. In pure water, however, even at low temperature, muscle fibres preserve their full contractility only for a short time.

Satisfactory results were obtained by employing a 50 per cent solution of glycerol. The fibre bundles, once extracted, can be preserved for weeks in this solvent at − 20° C. with undiminished contractility. The psoas was decomposed *in situ* into fibre bundles of about one millimeter in diameter. A thin stick was laid alongside, and four or five such bundles were tied to it at both ends and cut out. In this way straight fibre bundles of rest length and attached to the stick were secured. If bundles of equilibrium length were desired, only one end of the bundle was fixed to the stick and the other end cut, whereupon the muscle contracted to its equilibrium length. Then the other end of the bundle was fixed to the stick. In order to measure the difference between rest length and equilibrium length, a ligature was put on the free end of the bundle before cutting it, and the distance between the two ligatures was measured before and after cutting.

The bundle, tied to the stick, was placed into 50 per cent glycerol of 0° for twenty-four hours. Then the two ends of the muscle with the ligatures were cut off, whereby the muscle, detached from the stick, fell into the single bundles. The muscle was left in this condition for another day at 0° in 50 per cent glycerol and then transferred in this solvent into the deep freezer kept at − 20° C.

[4] Threads prepared from actomyosin are unfit as material for any experiment in which tensile strength is involved, since on extraction the continuous actomyosin filaments present in muscle are broken up, and actomyosin threads contain only their fragments. As will be shown later, one of the actions of ATP is to enable the actomyosin particles to slip alongside one another. Therefore, if an actomyosin thread is loaded or subjected to tension, and ATP is added, the actomyosin particles will contract, as they do in muscle, but they will also slip, and in spite of the contraction (observable in unloaded threads), the system will lengthen. This lengthening has led Buchtal, Deutsch, Knappeis and Petersen (1947) as well as Astbury, Perry and Reed (1948) to the erroneous conclusion that phenomena in actomyosin threads are fundamentally different from those in muscle.

The muscle in 50 per cent glycerol is too stiff to be decomposed into smaller bundles without straining, which causes the bundles to curl up. In water the muscle is too soft. For this reason, before the experiment, the bundles were transferred from 50 per cent glycerol to 20 per cent glycerol for an hour or so and decomposed here to the desired diameter, mostly into strips of 0.2 – 0.5 mm. diameter.

The psoas is built of smaller fascicles, and it is well to follow the outlines of these preformed bundles in decomposing the muscle. The dissecting was done by means of a pair of fine tweezers, used by watchmakers. The ends of the bundles are caught with these tweezers. By pulling them apart, the bundles can readily be separated. Before being subjected to experiment, the fibre bundles were examined under the microscope for continuity.

The experiments were performed in Ringer containing 0.001 M $MgCl_2$. In all experiments glass-distilled water was used because of the deleterious action of copper usually present in common distilled water.

Glycerol-treated fibre bundles of rest length and of 0.1 – 0.2 mm. diameter, if placed into a 0.25 per cent ATP solution, contract rapidly. Diffusion being the limiting factor, the rate of contraction depends also on the diameter. Unloaded fibres contract at room temperature to one-fourth or one-fifth of their rest length. If connected to the isometric lever, on addition of ATP they develop tension comparable in intensity to that developed by intact muscle on maximal excitation. If loaded they will also lift weights isotonically, similarly to intact muscle fibre bundles of similar dimensions.

This contraction of glycerol-treated muscle fibres under influence of ATP is one of the most striking biological phenomena and is very suitable for classroom experiments. Instead of ATP a freshly prepared boiled muscle juice may be used, or an ATP solution may be used prepared by elution of dried, alcohol-precipitated muscle. A smallish rabbit will provide material for a big class. Most of the experiments reported here were done by simple means and are suited for classroom experiments. Some of them have been repeated by the physiology class at the Marine Biological Laboratory at Woods Hole.

In several experiments the maximal total amount of work had to be measured. Theoretically, this can be done in the following way: The muscle is connected to the isometric lever, made to contract, and the tension is measured. Then the muscle is allowed to shorten slightly and made to contract, and the tension measured, etc., till the muscle has contracted maximally and develops no more tension. If the length is plotted against tension, the area between length and tension represents the total amount of work. Such an experiment was performed on the frozen sartorius after thawing. Its result is schematically reproduced in Figure 3, where the hatched zone is the total amount of work. This experiment is a rather difficult one and can be performed only with limited materal and under specific conditions. In the rabbit psoas, contractility is lost after thawing even at 0° C. in fifteen minutes, which makes the experiment impossible. In the frog the experiment can be done at low temperatures only, contractility being lost rapidly at higher temperatures.

A simpler method had to be found which could be applied in any material in a wider range of temperatures. Two such methods are suggested by Figure 3. In this figure the total amount of work is equal to the area BDF, which is one-half of the area BDFH, and the double of the area CDEG. Accordingly, we could measure the total amount of work in two different ways: (*1*) by measuring the maximum tension developed (DF) and multiplying it by the amount of maximum shortening

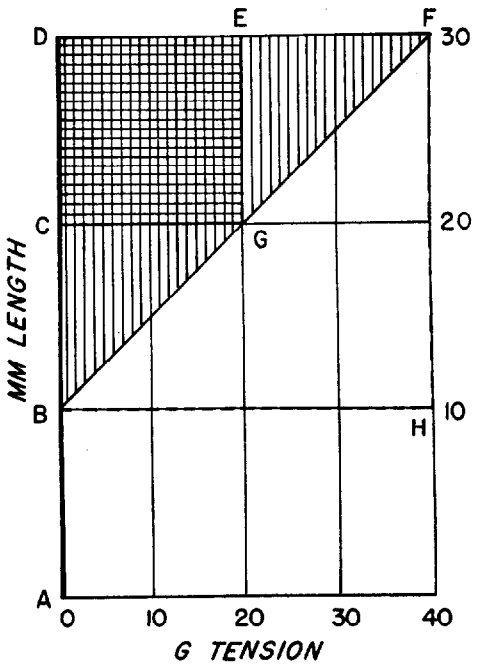

FIGURE 3. Schematic representation of muscular contraction. (Sartorius of the frog. Temp. 2.5° C.) AD = initial rest-length, DF = maximum tension, AB = length of maximally contracted muscle, unloaded. Area BDF = total amount of work. BF = length-tension diagram of the excited muscle.

of the unloaded muscle (BD), then dividing the result by two. Psoas strips, after freezing and thawing, contract at room temperature by two-thirds of their rest length. The formula will thus be $\frac{2/3\ lt}{2} = 1/3\ lt$ (l = rest length, t = maximum tension). A. V. Hill (1913) developed a similar formula which in his case (frog muscle excited electrically at 0°) was 1/6 lt. (*2*) Load the muscle with the weight corresponding to one-half of the maximum tension (DE), measure the distance by which the weight is lifted (EG), and take the double of the product of these two magnitudes (the area CDEG). The product will be the biggest if the weight is just one-half of the maximum tension, but a small deviation from this value will not cause a considerable error making the area bigger in one dimension and smaller in the other.

The first will be called the "isometric," the second, the "isotonic" method. Both methods may be criticized as to their exactness. The object of the present research is not to obtain exact numeric values, but to obtain information about the basic truth of the theory outlined.

PART II

Observation on heat contracture

According to Figure 1, the $\triangle$ F, i.e., the free energy spent by the single units, rises with increasing temperature. The free energy of the phosphate bond in ATP

is 11,000 calories (Meyerhof, 1944) and according to the theory discussed, this energy is needed for relaxation. According to the curve in Figure 1, the expenditure of energy in contraction reaches 11,000 calories at 47° in the frog and at 53° in the rabbit, and exceeds 11,000 calories above these temperatures. If the theory is correct, therefore, the muscle should be unable to relax at these temperatures and should persist in the fully contracted state.

In the frog, experiments were performed in the following way: the sartorius of *Rana pipiens* was provided with a ligature at both ends, was excised and loaded in one series with 2 g. (Fig. 4, circles) and in another series with 20 g. (triangles). The length between ligatures was measured and the muscle dipped into Ringer solution of varying temperature. Above 40° a rapid contraction ensued which was measured at the end of the second minute. The contraction reached its maximum at 47° C. The muscle remained in this maximally contracted state. The gradual lengthening at higher temperatures is due to the denaturation which takes place above 47° C. rather rapidly.

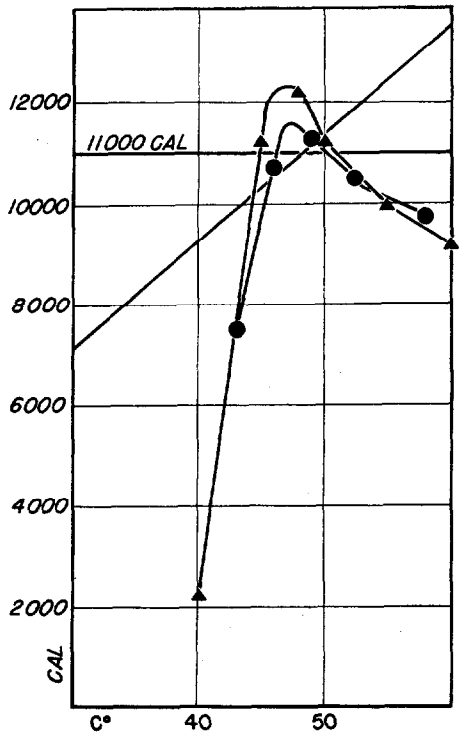

FIGURE 4.

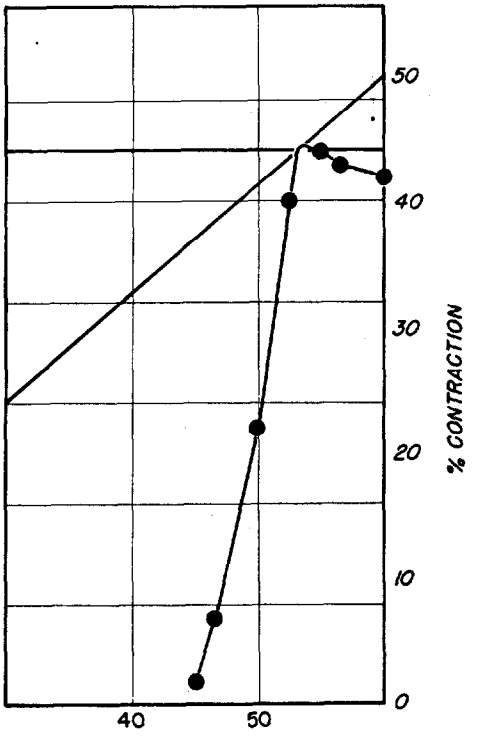

FIGURE 5.

FIGURE 4. Heat contracture in the frog sartorius. The coordinate net corresponds to the right-hand side of Figure 1. The sloping straight line is the △ F curve, and corresponds to the left-hand side ordinate. The points mark per cent of shortening and relate to the right-hand side ordinate of Figure 4. Abscissa: temperature in centrigrades.

FIGURE 5. Same as Figure 2. Strips of the musculus gracilis of the rabbit.

In the rabbit, experiments were performed in a similar way with the smaller weight (Fig. 5). Experiments were performed with strips of the musculus gracilis similar in dimensions to the frog sartorius. These strips were cut parallel to the fibres. The experiment was performed soon after the animal's death. The results were similar to those obtained in the frog. In both cases the muscle went into permanent maximal contraction at the temperature where the $\triangle$ F curve cuts the 11,000 calorie level, as demanded by the theory.

The fact that maximum and permanent contracture was reached only where the expenditure of F was 11,000 shows that the transference of energy from ATP to the contractile system goes without considerable loss.

This heat contracture must not be confused with the shortening of muscle due to heat denaturation. If a muscle is immersed into Ringer of 70° C. an extensive shortening is produced which is not due to the mobilization of the normal mechanism of contraction, but to denaturation. The difference between the two processes can easily be demonstrated. If the muscle is stored a few hours after death at room temperature or overnight in the ice box, the ATP disappears. No rapid contraction will be obtained at 53° in this muscle, but shortening will still be obtained at higher temperatures at which rapid denaturation is produced. This denaturation manifests itself, also, by a turbid appearance. The basic difference between the contraction obtained in the presence of ATP at 53° in the rabbit or 47° in the frog, due to the mobilization of the normal mechanism of contraction, and the shortening produced at higher temperatures and due to denaturation, can be demonstrated, also, by connecting the muscle to

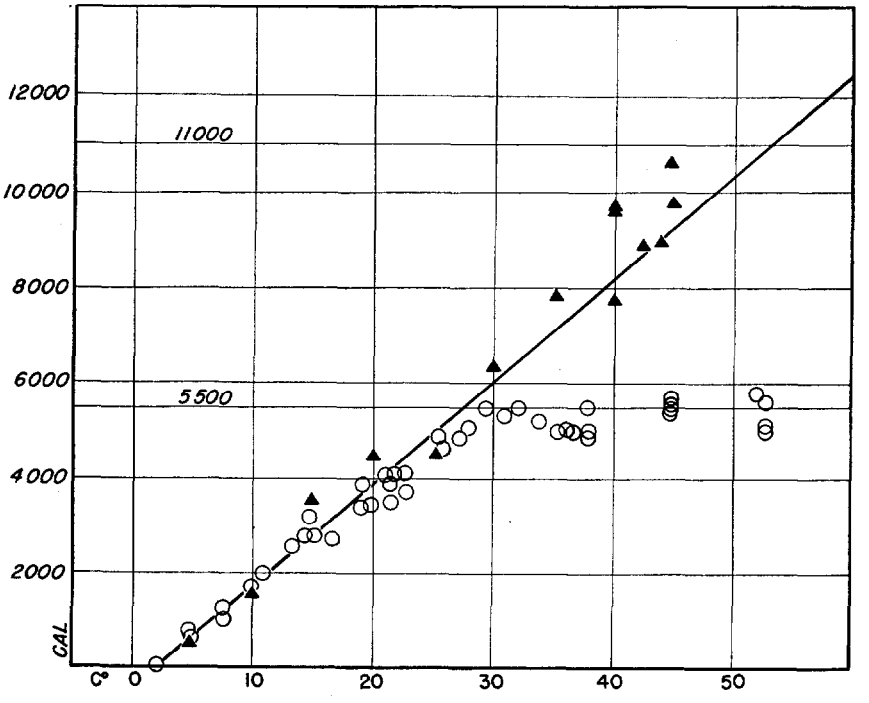

FIGURE 6. The work performed by strips of rabbit psoas, after freezing and thawing, at varied temperature, calculated for 35,000 gm. myosin present. The coordinates are identical with those of Figure 1. The sloping straight line is the theoretical $\triangle$ F curve. Triangles: isotonic measurement. Circles: isometric measurements.

the isometric lever. While the theoretical maximal tension is produced in the first case, scarcely any tension is developed in the latter. The gradual lengthening of the loaded muscle above 53 resp. 47° C. is evidently due to the denaturation. This denaturation in the rabbit sets in very rapidly above 53°.

Total work in the psoas

Free energy being, by definition, that amount of energy which can do work, the most direct way of testing our △ F curve is the measurement of the total work at different temperatures.

The amount of work was measured by both the isotonic and the isometric method and the results are reproduced in Figure 6. The psoas strips were loaded or connected to the isometric lever in the frozen condition, a moderate tension being given to the lever. The work done was expressed in calories and calculated for 35,000 g. of myosin. In these calculations the average myosin content of muscle (8 per cent) was taken into account, though it is probable that owing to poverty in connective matter, the psoas contains somewhat more myosin. In order to find out the quantity of myosin present, the muscle was weighed immediately after the measurement was finished, its ends with the ligatures having been cut off.[5]

The results of the isotonic experiments are marked in the figure with triangles. No measurements could be taken above 45° C. owing to the great sensitivity of the muscle to high temperatures after freezing and thawing. As will be seen, the agreement with the △ F curve is satisfactory and pleads for the basic truth of the theory.

The values obtained in the isometric measurement are marked with circles. As the figure shows, at lower temperatures the agreement of the experimental values with the △ F curve is satisfactory. This is true, however, only up to the 5500 calorie level, at which it shows a break to become parallel to the abscissa. This means that the maximum of tension is reached at 28° C. and increases no more if the temperature is raised.

This inability of the muscle to produce the expected tension at these higher temperatures is one instance of the so-called Fenn effect, named after its discoverer who found (1923) that the work done by the muscle depended also on the sort of job the muscle had to do.

The isometric method of calculating total work is based on the assumption that the length-tension diagram is a straight line. It is evident that in this region, where the isometric and isotonic curves differ, the l-t diagram cannot be straight and the method cannot be used. If the results calculated by this method are reproduced, this is because they nevertheless show the fact that on raising the temperature the tension developed remains constant.

The actual l-t diagram of the psoas can be found in this region by loading the frozen muscle strips with different weights, bringing them to the temperature in question, and measuring the maximum distance to which the weights are lifted. The results of such an experiment, calculated for the same weight of muscle, are reproduced in Figure 7.

The △ F curve of frog muscle

Varga's △ F curve admits but a very small expenditure of energy for the frog muscle at 0° C. (600 cal. of the 35,000 gm. unit), which means that at this tem-

[5] After freezing and thawing, contracted muscle rapidly loses weight by pressing out water. This loss may exceed 30 per cent, and is in agreement with the assumption that contraction is connected with loss of charge and hydration.

perature this muscle should be capable only of very feeble motion. It means, also, that the efficiency of the muscle would be exceedingly low because every autone would have to split one high-energy phosphate and pay 11,000 calories for the 600 calories spent.

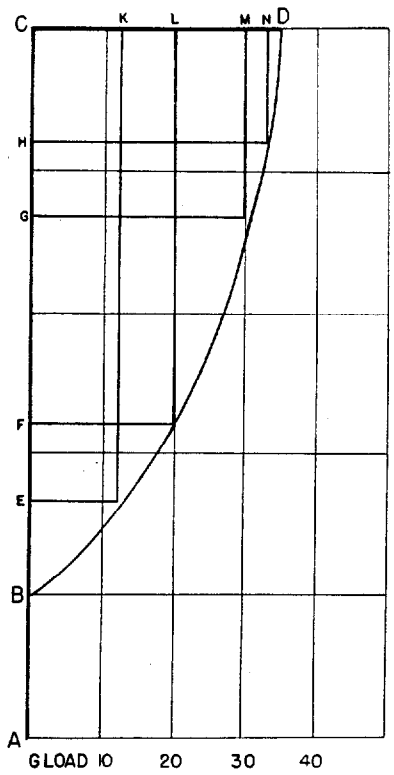

FIGURE 7. Length-tension diagram of strips of the psoas excited by freezing and subsequent thawing at 40° C. AC = length of the muscle; CH, CG, CF and CE are shortening with the weight CN, CM, CL and CK. The curved line connecting the corners of the squares (representing work done) is the l-t diagram.

As pointed out by A. V. Hill in the discussion following the author's lecture at the International Physiological Congress at Oxford (1947), frog muscle at 0° C. is capable of rather strong motion if excited strongly by direct stimulation. Hill had shown previously (1913) that not only is the tension developed by frog muscle at 0° C. rather high, but also the efficiency, which reaches 40 per cent (1939). The author was able to convince himself of the correctness of A. V. Hill's statements. Results of a few experiments on this line are reproduced in Figure 8. They show that the muscle actually spends much greater amounts of energy than allowed by the $\triangle$ F curve. The average expenditure around 0° was found to be 4500 calories, which corresponds to a 40 per cent efficiency if the 4500 calories are paid for by

the 11,000.[6] This agreement with Hill's results shows that freezing and thawing yields results similar to electric excitation.

The reason for the discrepancy between Hill's and Varga's results obviously was to be sought in the different nature of the material. Hill worked with intact muscle, Varga with muscle extracted with distilled water and frozen, or with actomyosin threads. It is easy to believe that in the animal the behavior of the actomyosin system is adapted to life at low temperature by some sort of regulation no longer present in extracted muscle or actomyosin.[7] This assumption would become acceptable if it could be shown that the $\triangle$ F curve of the whole muscle can be tranformed into the type of Varga's curve by substances which are known to abolish physiological regulations.

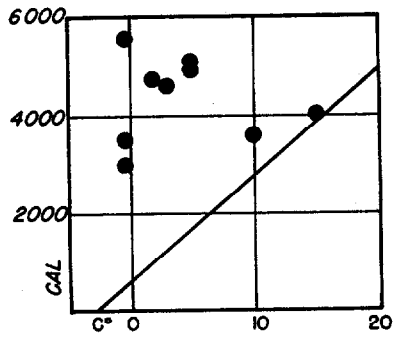

FIGURE 8. Work performed by the frozen sartorius after thawing, calculated for 35,000 gm. of myosin present. Coordinates correspond to lower left corner of Figure 1. Sloping straight line: $\triangle$ F curve.

Narcotics, at high concentration, inactivate many physiological mechanisms. Most of them also damage the contractile matter. Chlorated paraffins, however, like ethyl-chloride or chloroform, have no harmful action on actomyosin.

The sartorii of the frog (*Rana pipiens*) were exposed, and provided at their ends with ligatures; the distance between ligatures was noted. The muscle was excised, fixed at its original length and placed for five minutes in Ringer of 0° C., saturated with chloroform. Then the muscle was covered with freshly powdered dry ice and frozen. Its working capacity was measured in the isotonic and isometric experiment at varied temperatures. Also, the Ringer in which the muscle was made to thaw and contract was saturated with chloroform. If the work was measured above 15° C., the muscle was connected to the lever or the weight and allowed to thaw first in Ringer of 0° C., saturated with chloroform, and then transferred to the Ringer of higher temperature.

[6] When the work done by the right and left psoas was compared at slightly varied low temperatures (e.g. 0° and 3° C.), the muscle was found to have a greater $\triangle$ F at higher temperature; the slope of the resulting $\triangle$ F curve cut the abscissa at about $-30°$ C., and the 11,000 cal. level slightly under 47°. This suggests that the $\triangle$ F curve of intact frog muscle is similar to the curve of actomyosin, but has a different slope.

[7] The experiments were performed between November and January, and thus winter frogs were used.

The work done, and thus the free energy spent, was calculated for 35,000 gm. of myosin. The results are reproduced in Figure 9. The isotonic measurements could not be extended further to the right, above 40° C., the frozen muscle being damaged rather readily by higher temperatures. The action of the chloroform is reversible, and if the chloroform is washed out the work done at lower temperatures increases.

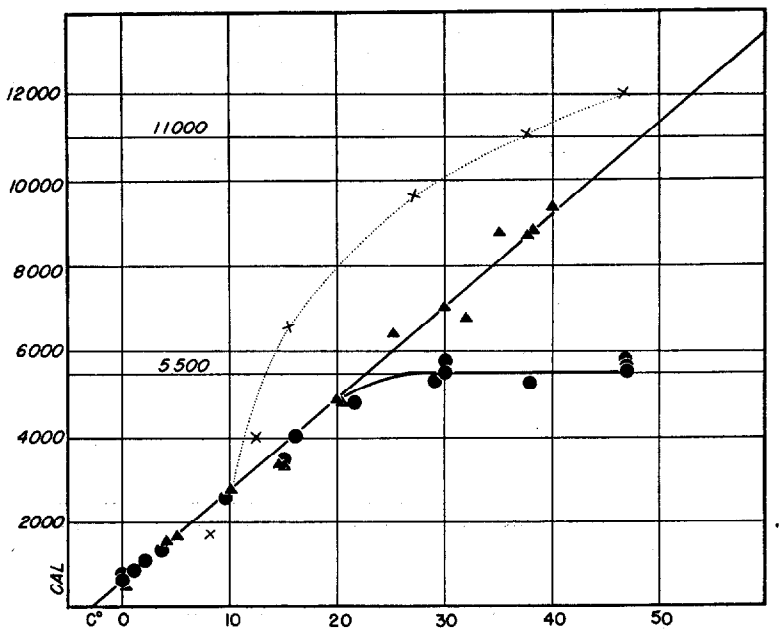

FIGURE 9. Work performed by the sartorius of *Rana pipiens* under influence of chloroform. The curve is analogous to Figure 5. Crosses: isotonic experiment with weights applied prior to freezing.

As the figure shows, the results obtained are similar to those obtained in the rabbit; the curves have the same shape but lie 5° lower. Here, again, the isotonic curve becomes asymptotic on reaching the 5500 calorie level.

In a series of experiments the muscle was loaded prior to freezing with the weight it was to lift later. The results are marked in Figure 9 by crosses connected by a dotted line. No correction was made for the elastic tension, but this correction would be smaller than the actual deviation from $\triangle$ F curve, which suggests that the expenditure of energy depends also on the tension which has to be overcome. It was observed repeatedly that the sartorius developed higher tension after freezing and thawing if stretched previously for a short while.

To sum up the experience with frog muscle, we can state that the $\triangle$ F curves of whole frog muscle and extracted frog muscle are different, the latter being identical with the $\triangle$ F curve of actomyosin threads. By treatment with chloroform, the curve of the whole frog muscle can be transformed reversibly into a curve similar to that obtained by Varga in his extracted material. This brings out the point

that the Δ F curve of actomyosin can be greatly modified by accompanying substances, and opens the possibility of adapting the contractile material to different physiological functions. Actomyosin is not a sharply defined substance and is accompanied by other different substances, proteins and lipins which actually make part of the system, and it is not surprising to find that systems containing different substances may have different Δ F curves. Ionic equilibria, disturbed by extraction, may also contribute to shaping the Δ F curve.

In the glycerol-treated psoas, immersed in 0.2 per cent ATP dissolved in Ringer, the Δ F curve, obtained by the isometric method, cuts the abscissa if extrapolated at $-10°$ C., while if extrapolated towards high temperatures, it cuts the 11,000 calorie level somewhat above 50° and has thus a different slope than untreated muscle.

Thermodynamic reversibility

One of the most important implications of the Δ F curve (Fig. 1) is the thermodynamic reversibility of contraction, which means that the energy spent by the single units in contraction is a function of temperature on which it depends in a reversible way. It should thus be possible to increase or decrease the tension of the contracted muscle solely by variation of the temperature.

Owing to secondary complications, not every material is suitable for this demonstration. The rapid loss of ATP and contractility in the rabbit muscle, after freezing and thawing, rules out this material. In the intact frog muscle, as shown, the effect of temperature is compensated. We can expect to be able to demonstrate

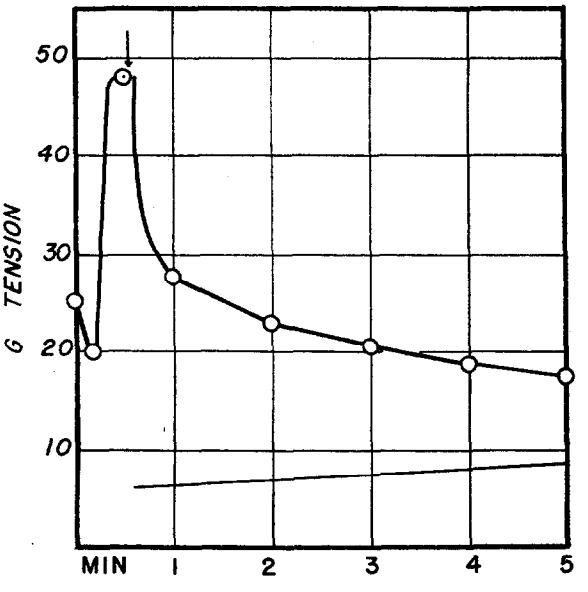

FIGURE 10. Tension developed by the frog muscle, treated with chloroform, at 13 and 0–1° C. At the arrow the warmer Ringer was substituted by the cold one. Sloping straight line: tension demanded by the Δ F curve.

thermodynamic reversibility in the frog muscle treated with chloroform, or in extracted strips of the psoas immersed in a solution of ATP.

Figure 10 illustrates a result obtained with frog muscle. The sartorius was treated with chloroform and frozen, connected to the isometric lever and dipped into chloroform Ringer at 13° C. After a short negative phase, usually seen in such conditions, the muscle rapidly contracted developing 48 gm. of tension. As the maximum was reached (26 sec.) the Ringer was exchanged for another chloroform Ringer of 0° C. The muscle suddenly relaxed. During the experiment the temperature of the Ringer rose 1° C. The sloping line in Figure 10 shows the theoretical tension of the muscle demanded by the ΔF curve. As can be seen, the tension of the muscle asymptotically approaches this line. The control experiment done with the other sartorius of the same frog showed that if the temperature is kept constant at 13° C., the tension remains high after a slight initial depression and does not fall more than a few per cent in five minutes.

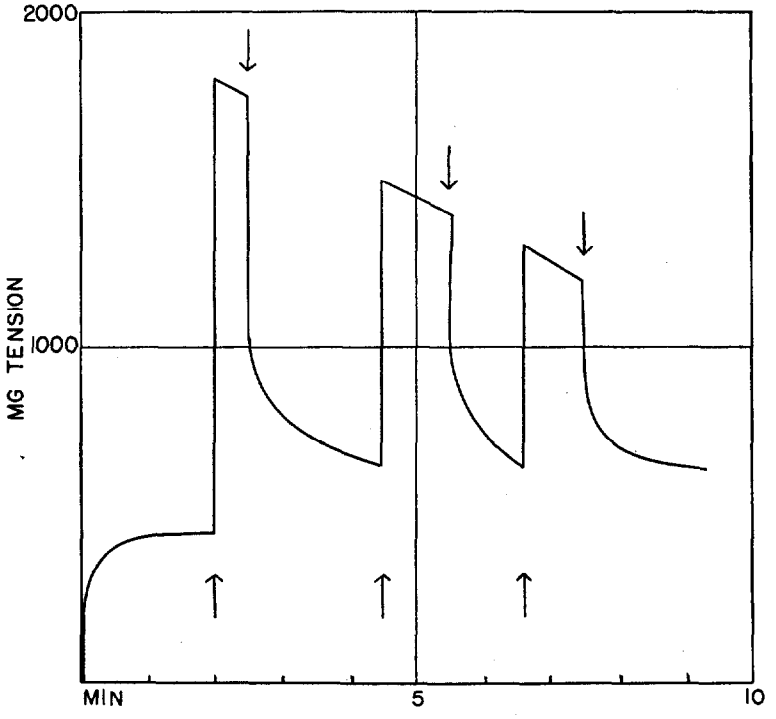

FIGURE 11. Tension developed by extracted psoas fibres in ATP at 25° and 1° C.

The following experiment, reproduced in Figure 11, illustrates thermodynamic reversibility in a fibre bundle of the psoas, treated with glycerol: The fibre bundle (42 mm. long and 0.5 mm. thick) was connected to the isometric lever, a tension of 200 mg. was given, and the muscle immersed in Ringer of 1° C. Then the Ringer was exchanged for another Ringer of the same temperature containing 0.2 per cent ATP. The tension rose to 450 mg. At the arrow pointing upwards, the

fluid was exchanged for an identical solution of 25° C. The tension was noted at once and found to be 1800 mg. A reading was taken every thirty seconds and the warm ATP Ringer was exchanged for the cold one (arrow pointing downwards). A reading was taken at once and subsequently every thirty seconds. At the arrow pointing upwards, again, the warmer Ringer was introduced, etc. As can be seen in the figure, the change in tension is immediate and reversible. As the experiment went on, the muscle gradually lost contractility.

The measurement of the diameter has no pretense of accuracy. If the tension is calculated from the final measurement at 25° for one cm.^{2}, a tension of 2½ kg. is obtained, which shows that the tension developed by a glycerol-extracted muscle under the influence of ATP is of the same dimension as the tension developed by an intact muscle under the influence of maximal stimulation.

PART III

Elasticity of the resting psoas

The fresh, resting psoas shows a moderately high elasticity, as demonstrated by the following experiment (Fig. 12): A strip of the psoas of the freshly killed rabbit was connected to the isometric lever, weight 56 mg., rest length 78 mm. (RL in fig.), equilibrium length (EL) immediately after excision, 62 mm. The muscle was slowly stretched, its length being increased by one millimeter in five seconds. After the rest length was reached, the muscle was released for a few seconds and then its equilibrium length measured. This was done by straightening the muscle out, measuring its length, and then applying a tension of 200 mg. and measuring the length again. The difference in length in both measurements was usually 2 mm. In the figure the average of these two measurements is given. After this measurement was completed, the muscle was stretched to the length from which it was released. This stretching was roughly twice as fast as the stretching before. Then the muscle was stretched further at the original lower rate and the procedure repeated after every 5 mm. of additional stretching till the muscle broke. The muscle was kept during the experiment in a wet chamber, immersed in a water-bath of 0° C. In the figure the gradual stretching is symbolized by the upper straight line which refers to the ordinate (mm). The corresponding equilibrium length is reproduced in the middle curve. The single points of this curve lie under the point of the upper curve from which the muscle was released. The lowest curve shows the tension developed on stretching and refers to the ordinate, the numbers of which mean gram-tension in this case. The lowest straight line simply shows a slope of 45° and means that if the curve of tension is parallel to this line, the muscle obeys Hooke's law.

The upper line shows that the muscle broke when extended to 173 per cent of its equilibrium length, and the middle curve shows that this extension was elastic in the whole range of measurements. The middle curve illustrates the well known fact of the poor reproducibility of the equilibrium length. The lowest curve shows that up to the rest length the contracted units can be stretched practically without resistance, but begin to develop resistance at this point. If the muscle were *in vivo* at its equilibrium length, it could develop no tension at the beginning of contraction; if it were tensed any more, it would be spastic.

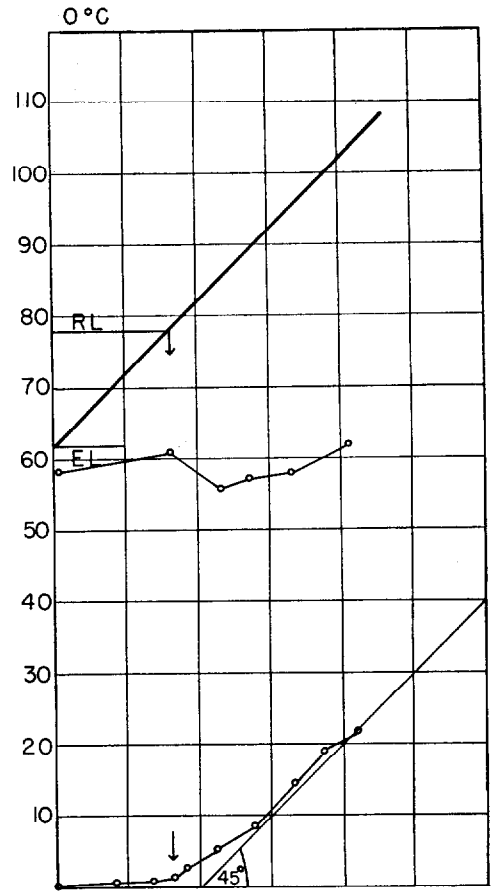

FIGURE 12. Length-tension relations of the psoas at 0° C. (see text).

In Figure 13 an identical experiment is reproduced, performed at 23° C., weight of the muscle 130 mg.

In this experiment, the muscle broke when extension reached 190 per cent of the equilibrium length. As the middle curve shows, this extension is, at its higher degrees, not completely reversible, and the elastic part of the extension is but 163 per cent of the equilibrium length. The middle curve shows the rest length to be better reproducible at this temperature. The lowest curve again shows the contracted units to be extensible at the beginning with practically no resistance. The middle part of the curve obeys Hooke's law; the upper part shows excessive tension. The transition from the region of low tension into the Hooke region is rather sharp and corresponds to the rest length. If the relaxed units contract, the tension developed will be proportional to the contraction from the beginning which makes precise motion possible. On the other hand, having practically no tension, they will not impede the motion of their antagonist.

The muscle obeys Hooke's law up to one-half of the maximum of tension. At the point where it begins to develop excessive tension, stretching begins to be inelastic, causing slipping and permanent damage to the muscle.

Elasticity, ATP, and the slope of the ΔF curve

Freshly isolated strips of the psoas show high elasticity. After the death of the animal, its ATP gradually disappears in a few hours' time, as shown by M. Borbiro in a separate paper (pp. 162–7, this issue). Parallel to this disappearance of ATP, the elasticity of the muscle declines, and if the muscle is excised four hours after death, it will usually be found entirely inelastic. On stretching, the maximum tension is developed at once, and the muscle tears without considerably increasing its length.

The question arises whether the high elasticity of fresh muscle is actually due to the ATP present, and whether the disappearance of this elasticity can actually be attributed to the decomposition of this nucleotide. It can be shown that this is

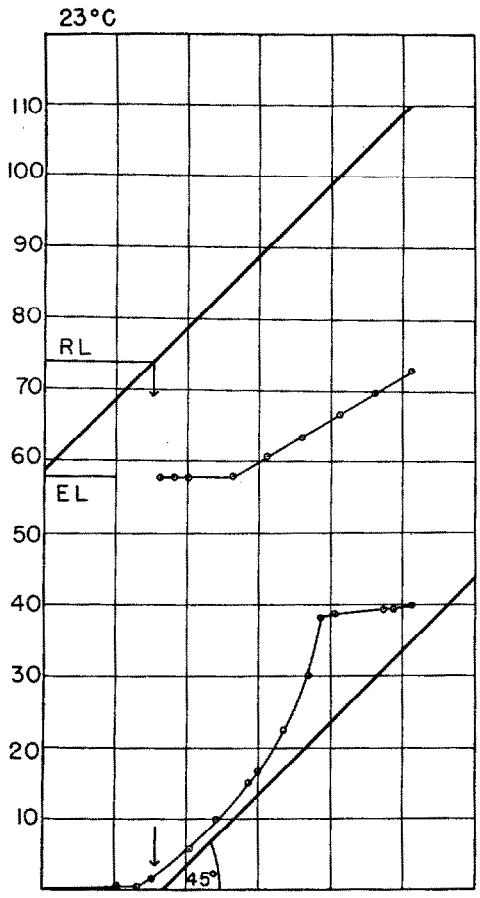

FIGURE 13. Same as Figure 12, at 23° C.

actually the case: if strips of the psoas are extracted at equilibrium length with 50 per cent glycerol, they are found to be entirely inelastic. At 0°, in Ringer, they cannot be stretched at all without breaking, and even at 13° C. extensibility does not exceed two per cent. If, however, 0.2 ATP is added to the Ringer, the muscle again becomes extensible. Using fibre bundles of 0.3–0.4 mm. diameter, the muscle could readily be stretched at 0.5–1° C., on an average to 145 per cent of its rest length.[8]

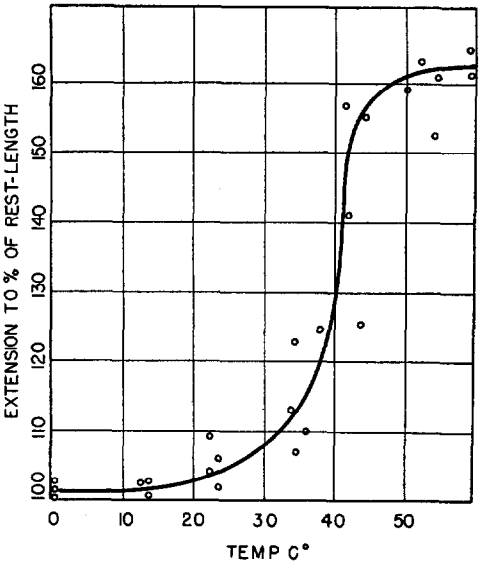

FIGURE 14. Extensibility of extracted psoas fibres at varied temperature. 100 per cent of rest length (abscissa) means that the fibres are not extensible.

The extensibility of the extracted muscle, in absence of ATP, is a function of temperature (Fig. 14). At 0° the muscle is practically not extensible; extensibility rises slowly with increasing temperature, rising rapidly at body temperature. The extensibility at higher temeperatures, up to 53°, is not due to denaturation, as shown by the relatively big force needed for extension. Denaturation rapidly sets in at a somewhat higher temperature, 54–55°, where the muscle soon becomes plastic, offering practically no resistance to stretching.

This shows that, in absence of ATP, the actomyosin particles in muscle are surrounded by unbalanced forces which link neighboring particles together and make it impossible for them to move relative to one another, turning the system into a rigid, inelastic gel. They are counteracted by heat-agitation.

The extensibility of muscle at low temperatures in presence of ATP shows that the elasticity of muscle actually depends on its ATP, which must be present in the resting state linked to the actomyosin. The interdependence of ATP and elasticity also shows that in the psoas the elastic properties observed were predominantly

[8] These results are in agreement with previous findings of Th. Erdös (1943) on the relation of ATP to rigor mortis.

those of the contractile matter, actomyosin, and were not due to the sarcolem or the connective tissue present, no specific reaction being known to take place between these latter and ATP.

It is evident that free energy is needed to abolish the cohesive forces linking the particles together, and if this is achieved by ATP, so it is also evident that part of the free energy of the ATP-actomyosin system will have to be spent in this reaction. The free energy spent in this reaction will manifest itself in the stability of the link between ATP and the actomyosin. The free energy needed to make actomyosin elastic will decrease with increasing temperature, actomyosin becoming more elastic by itself on elevation of the temperature. This expectation is in agreement with the results of Mommaerts (1941–1942) who found that at low temperatures, the dissociation constant of the actomyosin-ATP complex was exceedingly low. F. B. Straub (1941–42) found that the binding of pyrophosphate to actomyosin greatly depends on temperature, being strongest at the lowest temperature.

We can thus conclude that the free energy of the actomyosin-ATP system is spent in two successive steps. In the first step the ATP is linked to the myosin, cohesive forces are abolished, and a new system is formed in which particles are rendered capable of relative motion, contraction or extension. The free energy spent in this reaction, henceforth called "Reaction I," will decrease with increasing temperature.

The second step, "Reaction II," entails the dimensional change called "contraction." In the resting muscle, we find the actomyosin-ATP system in the state of Reaction I, but Reaction II is inhibited by some unknown mechanism. This inhibition is abolished by "excitation" which causes Reaction II to take place. In the glycerol or water-extracted muscle suspended in ATP this inhibitory mechanism is no longer present, and Reaction I is followed spontaneously by Reaction II.

It is evident that Reaction II can only spend the free energy unspent by Reaction I, which may be involved in the slope of the Δ F curve of contraction (Fig. 1).

Since actomyosin devoid of ATP-ase activity can still contract, as shown by Buchthal, Deutsch, Knappeis and Petersen (1947), we can conclude that no phosphate is liberated in Reaction I or II, and the whole loss of free energy of the system takes place without splitting of high-energy phosphate links.

The increase of extensibility of actomyosin under the influence of ATP was the first known specific effect of ATP on "myosin" discovered by Engelhardt, Ljubimova and Meitina (1941).

If actomyosin is stored in dehydrated condition, links are developed which are not quantitatively split by ATP. Such links develop especially fast in contracted, thus discharged, actomyosin. Their development is favored by parallel setting.

These observations on elasticity and its *post mortem* changes are in agreement with previous findings of Th. Erdös (1943), corroborated and extended by Bate-Smith and Bendall (1947).

The weight of the autones

The Δ F curve (Fig. 1) shows the free-energy change of the single autones at any given temperature. If the weight and myosin content (8 per cent) of the muscle are known and the Δ F curve and measurements of the total work are accepted, the weight of the single autones can be found by simple numeric calculation.

If, for instance, at a given temperature the $\triangle F$ curve indicates an expenditure of 5500 calories per unit, and our piece of muscle performed 0.0055 calories' worth of work and contained 35 mg. of myosin, then the weight of the myosin-unit which has spent 5500 calories would have been 35,000 gm., and this would be the unit weight of myosin contained in one autone. As has been shown (Figs. 6 and 9), isotonic measurements and isometric measurements up to the break indicated a unit weight of 35,000 gm. for myosin. Above the break, the isometric experiments do not yield correct values. At the temperature at which the $\triangle F$ curve cuts the 11,000 calories level (53° C. in the rabbit and 47° C. in the frog), the unit weight calculated from the work done by the isometric method must be the double of 35,000 gm. In a series of experiments the unit weight of myosin was calculated from the work done by the sartorius as measured by the isometric method at 47° C. The freshly isolated sartorius was in these experiments connected to the isometric lever and dipped into Ringer of 47° C. The results are given in Table 1.

TABLE I

	74,000
	66,000
	74,000
	74,000
	69,000
	70,000
	72,000
Average	72,000

This calculated unit weight of 35,000 gm. is based on the current myosin estimations. Should muscle be found to contain more myosin than 8 per cent, this would mean that the unit weight of myosin taking part in the building of one autone is correspondingly higher. There are indications suggesting that the psoas actually contains more myosin than 8 per cent. Moreover, if there is a loss of free energy, this also entails a bigger unit weight. So 35,000 gm. is rather an order of magnitude and the lower limit than the absolute value, which might be equally well 70,000 gm. H. B. Bull (1946) arrived along different lines at a unit weight of 40,000 gm.

CONSIDERATIONS

It may be asked how far the observations made on the psoas of the rabbit, a specific case, reflect a more general behavior. There are different kinds of muscle with widely different functions and structure. As reported before, the contractile matter of all these different muscles seems to be similar, and actin and myosin prepared from cross-striated, smooth, or heart muscle, or even myomas, can be interchanged to form actomyosin which contracts on addition of ATP. Even clam muscle shows similar reactions (A. Lajta, 1947).

There are indications suggesting that the regularities observed are not limited to the contractile matter. If the muscle is minced soon after death and suspended in an alkaline 0.6 M KCl solution, a sticky extract is obtained which owes its high viscosity to the dissolved fibrous structural protein, actomyosin. The hydration and dissolution of this protein is not merely a result of its interaction with the salt-solution. The ATP present has a decisive influence, and if we store the minced muscle for a few hours prior to extraction, giving time for the decomposition of ATP, the subsequent extraction will yield an extract of low viscosity containing no actomyosin. Addition of ATP will restore conditions found in fresh muscle.

As shown by Lajta (unpublished), kidney and other tissues behave in an analogous way. The fresh mince, if suspended in the alkaline salt solution, yields a sticky, highly viscous extract, and the strong double refraction of flow reveals the presence of dissolved fibrous structural proteins. If, however, the mince is incubated, the subsequent extraction yields a fluid of low viscosity containing no fibrous proteins. During the incubation the labile phosphate present disappears. Contrary to muscle, however, the original condition cannot be restored by the addition of ATP or a fresh boiled juice. The labile phosphate, the disappearance of which seems to be connected with this change, is found to be linked to nucleic acid present in the protein. The nucleic acid, prepared from fresh kidney, shows a high content of labile phosphate.

This behavior is completely analogous to that found in muscle, with the difference that instead of a single nucleotide, ATP, in kidney and other parenchymatous organs we find nucleotides united to long chains, to nucleic acid. In muscle, such long chains would interfere with motility.

The close analogy with muscle suggests that in other organs, too, the protein is built of small functional units, each correlated to a nucleotide which governs its physical state and enables the system to develop the two different states, the high-energy, charged, hydrated state corresponding to rest, and the low-energy level corresponding to activity.

Summary

Material and methods of measurement of physical properties of muscle were discussed.

Heat contracture, total work of muscle, and thermodynamic reversibility were studied and found to be in agreement with earlier assumptions.

Elastic properties of muscle and their relation to ATP were studied.

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